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Electronic Supplementary Information

Inducing mesenchymal stem cell attachment on non-cell adhesive hydrogels through click chemistry

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Experimental Section

Materials

Deacetylated gellan gum was obtained from Millipore Sigma (Billerica, MA). Ultrapure water (Milli-Q, 18.2 MΩ, EMD Millipore, Taunton, MA) was used in all experiments. Cation exchange resin Dowex® 50WX8 hydrogen form (50-100 mesh), sodium hydroxide, furfurylamine, Triton X-100, monoclonal anti-vinculin-fluorescein isothiocyanate (FITC) antibody, Dulbecco's phosphate buffered saline (PBS), 4',6-diamidino-2-phenylindole (DAPI), Tween 20, 2-(*N*-morpholino)ethanesulfonic (MES) acid hydrate, sucrose, sodium chloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), magnesium chloride hexahydrate, and low-glucose Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, MO). Paraformaldehyde was obtained from Electron Microscopy Sciences. Isopropanol, dimethyl sulfoxide (DMSO), Pierce dye removal column, Alexa Fluor 594 phalloidin, 4-(4,6-dimethoxy[1,3,5]triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM), normal goat serum (NGS), fetal bovine serum (FBS), and L-glutamine were purchased from Fisher Scientific (Hampton, NH). Penicillin–streptomycin and trypsin–ethylenediaminetetraacetic acid (EDTA) were purchased by Caisson Labs (Smithfield, UT). Regenerated cellulose dialysis membrane (50 kDa molecular weight cut-off) was purchased from Spectrum Laboratories, Inc. (Piscataway, NJ). A maleimide-modified RGD-peptide (Mal-GRGDS) was obtained from Genscript Biotech (Piscataway, NJ). Fibronectin HiLyte 488 was obtained from Cytoskeleton, Inc. (Denver, CO). Dibenzocyclooctyne (DBCO) amine, tetra-acetylated *N*-azidoacetyl-D-mannosamine (Ac_4ManNAz), carboxyrhodamine 110 DBCO, and carboxyrhodamine 110 azide were purchased from Click Chemistry Tools (Scottsdale, AZ). Human bone marrow-derived mesenchymal stem cells (MSCs) (33 year old female) were purchased from Lonza (Hopkinton, MA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies (Tokyo, Japan). Deuterated water was obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA). All chemicals were of analytical reagent quality or high performance liquid chromatography (HPLC) grade and used without further purification.

Methods

1. Functionalization of gellan

In this work we present a novel approach based on click chemistry where we covalently bind MSCs to a hydrogel to investigate the potential of this approach as an alternative way to

develop a cell adhesive material. For this purpose, gellan polymer was functionalized with DBCO and we compared our approach with a more conventional approach, i.e. RGD functionalization of gellan.

Synthesis of DBCO-gellan

Gellan gum was first purified following previously established procedures to remove and replace a majority of the cations in the polymer with Na^+ ions.[1] Briefly, 5 g of gellan were dissolved in 500 mL of water with continuous stirring at 60 °C. Next, 25 g of cation exchange resin was added to this solution and stirred for 30 minutes. The resin was removed by decantation maintaining the temperature at 60 °C, and 1 M sodium hydroxide was added to the gellan solution, until the solution reached pH 7. Gellan was precipitated in approximately 1 L of isopropanol under vigorous stirring, filtered, dried, re-dissolved in water, and dialyzed using a 50 kDa molecular weight cut-off regenerated cellulose dialysis membrane for 3 days at room temperature. The water was then removed by lyophilization. The cation content in the polymer before and after the purification process was determined by inductively coupled plasma optical emission spectrometry (iCAP 7400 ICP-OES DUO, Thermo Fisher, Waltham, MA).

DBCO was conjugated to the gellan in a single step reaction. Purified gellan was functionalized with DBCO amine (1:0.5 molar ratio gellan repeat unit to DBCO) using DMT-MM as a coupling agent (1:5 molar ratio DMT-MM to gellan repeat unit) in 50% v/v DMSO in water. The reaction was kept stirring at 40 °C for 4 hours, followed by purification via dialysis at room temperature against 25% v/v DMSO in water for one day and subsequently against water for two days, using a 50 kDa molecular weight cut-off regenerated cellulose dialysis membrane. The reaction yielded a DBCO functionalization of 45% as calculated by proton nuclear magnetic resonance (^{1}H-NMR), recorded in deuterated water using a Bruker Avance III Ultra-Shield Spectrometer, 400 MHz.

Synthesis of RGD-gellan

RGD-gellan was synthesized in a two-step reaction as previously reported.[2] Briefly, purified gellan was first functionalized with furfurylamine (1:1 molar ratio gellan repeat unit to furfurylamine) using DMT-MM as a coupling agent (1:5 molar ratio DMT-MM to gellan repeat unit) in water. The reaction was kept stirring at 37 °C for 24 hours, followed by purification via dialysis at room temperature against water for three days using a 50 kDa molecular weight cut-off regenerated cellulose dialysis membrane. The reaction yielded a furan functionalization of 65% as calculated by ^{1}H-NMR, recorded in deuterated water using a Bruker III Ultra-Shield Spectrometer, 400 MHz (data not shown). The furan-gellan was then further functionalized with Mal-GRGDS peptide (1:3 molar ratio gellan repeat unit to peptide) in a 50% v/v mixture of water and MES buffer (100 mM at pH 5.5). The reaction was kept stirring at 37 °C for three days, followed by purification via dialysis at room temperature against water for three days, using a 50 kDa molecular weight cut-off regenerated cellulose dialysis membrane. The reaction yielded a peptide functionalization of 45% as calculated by ^{1}H-NMR, recorded in deuterated water using a Bruker Avance III Ultra-Shield Spectrometer, 400 MHz (data not shown).

Reactivity of DBCO-gellan with an azide functionalized mannose

To establish the reactivity of the DBCO once attached to the gellan polymer, the reaction between DBCO and Ac_4ManNAz was monitored through ultra-violet (UV) spectroscopy using a Cytation™ 3 plate reader (BioTek, Winooski, VT) and liquid chromatography-mass spectrometry (Agilent 6530 LC-MS, Agilent, Santa Clara, CA). DBCO-

Gellan **1** or DBCO amine was mixed with Ac_4ManNAz at a molar ratio of 1:10 or 1:1, respectively, and the reaction was monitored over time for 24 hours by UV spectroscopy. The crude Man-DBCO **3** was analyzed via LC-MS to confirm the conjugation.

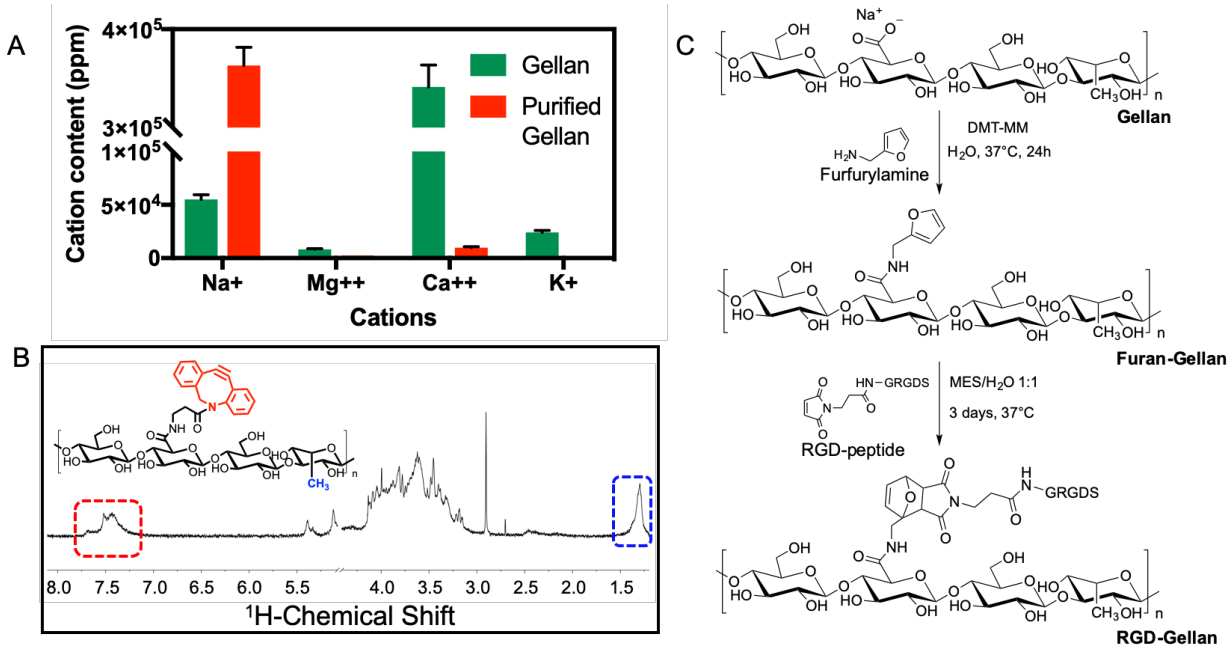

Figure S1. A) Quantification of the cations present in gellan before (green) and after (red) purification examined via ICP-OES. B) ^{1}H-NMR spectra of DBCO-gellan conducted in deuterated water (the solvent peak has been removed from the spectra). C) Synthetic scheme of gellan functionalization with RGD-peptide.

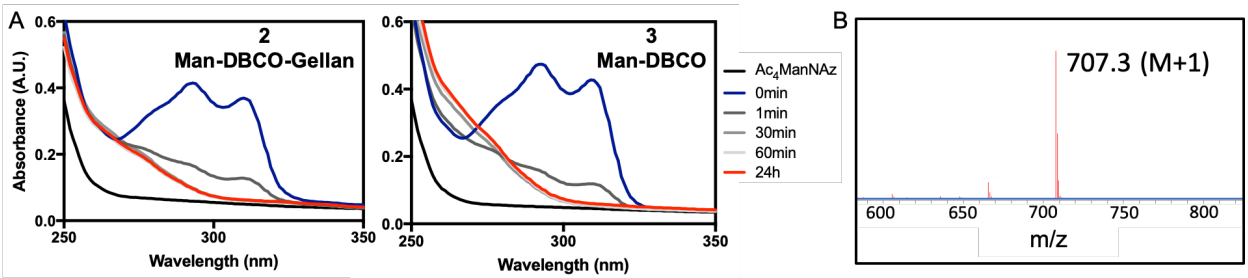

Figure S2. A) Absorbance spectra of conjugation reactions of compound **2** (left) and **3** (right) over time. B) LC-MS of compound **3** used as reference to monitor the reaction on gellan.

2. Hydrogel formation and characterization

DBCO-gellan was used to form functionalized hydrogels and their physical properties were investigated prior and after incubation in MSC culture media. These studies showed that the salts present in the media increased ionic cross-linking, indicated by a ~10-fold increase in Young's modulus for 2% w/v gellan hydrogels formulated with and without DBCO-gellan after incubation in MSC media along with increases in G' and G''. These hydrogels also demonstrated shear thinning, characteristic of gellan hydrogels.[3] Finally, a ~15% decrease in the mass of hydrogels incubated in MSC media was observed over 7 days.

Gellan hydrogel fabrication

All gellan hydrogels reported in this study were formulated with 2% w/v gellan (inclusion of DBCO- or RGD-gellan are noted). In order to prepare mixtures of gellan and DBCO-gellan for hydrogel fabrication, the two polymers were first dissolved separately. Gellan was dissolved in boiling water (100 °C), and DBCO-gellan was dissolved in 100% v/v DMSO at 37 °C. These two polymer solutions were combined in the appropriate ratios for hydrogel fabrication, with a final concentration of DMSO equal to 5% v/v. The final concentration of unmodified gellan was 2% w/v, while the concentration of DBCO-gellan was either 0% (these controls were also prepared with 5% v/v DMSO), 0.1%, or 0.25% w/v. For rheology, swelling analysis, and compression testing, 5 mL of the gellan mixtures were poured into 60 mm Petri dishes at 100 °C and allowed to cool to room temperature while setting. Cylindrical hydrogels (approximately 80 µL volume, diameter 5 mm and height 4 mm) were produced from this larger hydrogel using a biopsy punch and used for rheology, swelling analysis, and compression testing. For the *in vitro* cell experiments, the gellan mixtures at approximately 100 °C were poured directly into the wells of 96-well plates (100 µL for each well) or in cover-glass bottom 4-well Thermo Scientific™ Nunc™ Lab-Tek™ Chambered Coverglass (150 µL for each well) forming hydrogels upon cooling. RGD-gellan hydrogels were fabricated similarly to DBCO-gellan containing hydrogels; however, RGD-gellan was initially dissolved in a mixture of 50% v/v DMSO in water and not pure DMSO. RGD-gellan hydrogels were prepared at a final concentration of 2% w/v unmodified gellan and 0.1%, 0.2%, 0.5%, or 1% w/v RGD-gellan. All hydrogels formulations were prepared at a final concentration of DMSO equal to 5% v/v.

Characterization of gellan hydrogel physical properties

We investigated the Young's modulus, storage (G') and loss (G'') modulus, and swelling capacity of all hydrogel formulations. The Young's moduli of the gellan formulations in the absence or presence of 5 mL of MSC growth media was evaluated through compression testing at 23 °C using a Bose Enduratec ELF 3200 (TA Instruments, New Castle, DE) with a top indenter and a bottom parallel plate. Hydrogels were analyzed before and after exposure to MSC growth media at room temperature for 30 minutes.

Oscillatory rheology was used to examine the viscoelastic properties for hydrogels at 37 °C before and after incubation in 5 mL of MSC growth media at room temperature for 30 minutes using a TA Advanced Rheometer 2000 (TA Instruments, New Castle, DE). Applying 2% strain, a frequency sweep was carried out over a frequency range of 0.1 Hz to 10 Hz. G' and G'' were determined over this frequency range. Viscosity as a function of shear rate was also investigated for hydrogels before and after incubation in cell culture media. A shear rate ramp from 0.1 to 10 s^{-1} was carried out at 37 °C and viscosity was determined.

Finally, the swelling properties of all hydrogel formulations were examined. The initial mass of as-made hydrogels was assessed (M_o). Hydrogels were each incubated in 3 mL of MSC growth media at 37 °C for 7 days. The mass of these hydrogels was examined daily (M_t) and compared with the initial mass to determine swelling capacity using equation (S1).

$$Swelling\ Capacity = \frac{M_t}{M_o} \times 100\% \quad \text{(S1)}$$

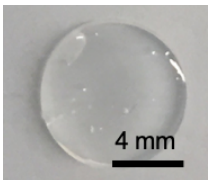

Figure S3. Digital photograph of gellan hydrogel containing 2% w/v unmodified gellan and 0.1% w/v DBCO-gellan.

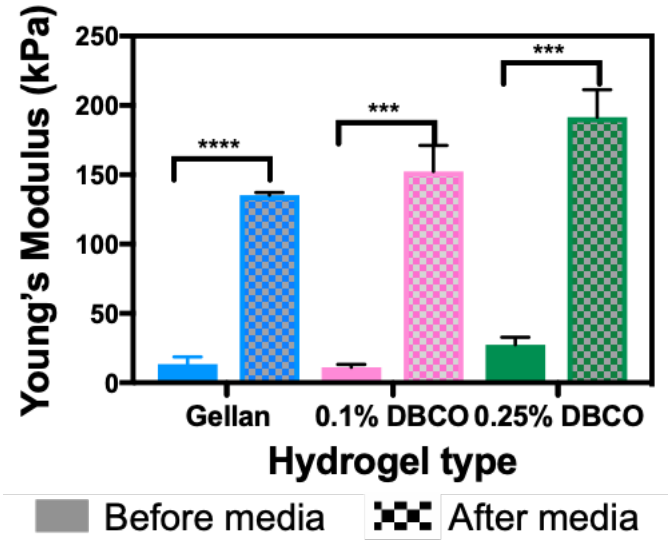

Figure S4. Young's modulus obtained via compression testing of 2% w/v gellan hydrogels with increasing concentrations of DBCO before (left bars) and after (right bars) incubation in MSC growth media for 30 min at room temperature. Data are shown as mean ± standard deviation. Statistical significance was examined using unpaired t-test (n = 3, ***p <0.001, ****p <0.0001).

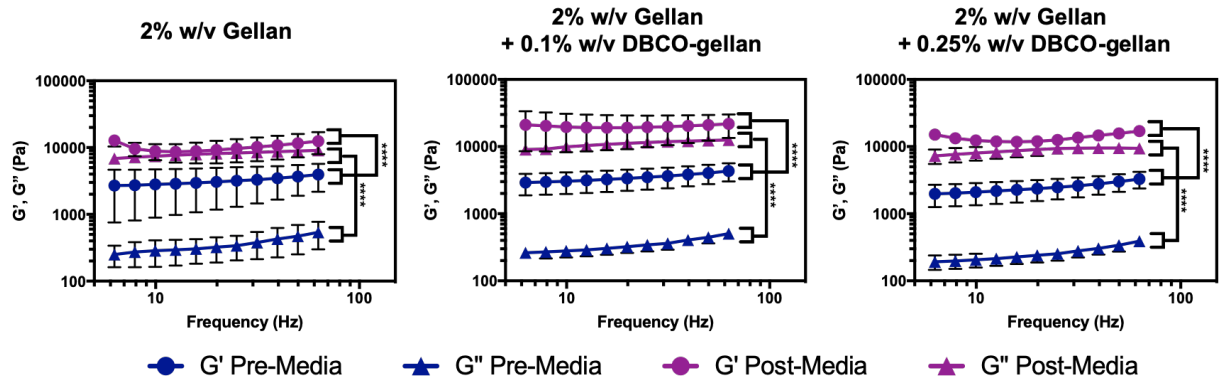

Figure S5. Storage (G') and loss (G'') moduli of gellan hydrogels before and after incubation in MSC growth media for 30 minutes at 37 °C over a frequency sweep of 0.1 to 10 Hz. Data are shown as mean ± standard deviation. Statistical significance was examined using two-way ANOVA (n = 3). Comparing pre- and post-media treatment, all formulations exhibited statistically significant differences (**** p <0.0001).

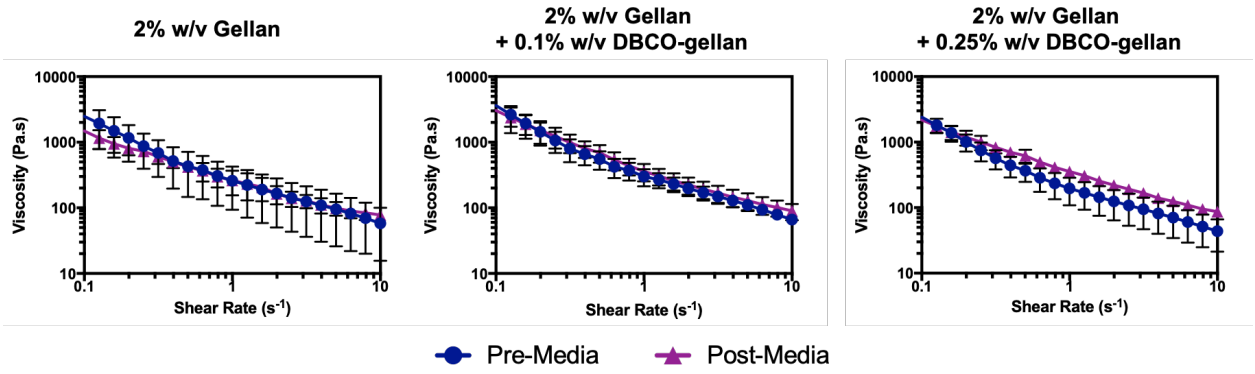

Figure S6. Viscosity versus shear rate for gellan hydrogels before and after incubation in MSC growth media for 30 minutes at 37 °C over a shear rate of 0.1 to 10 s^{-1}. Data are shown as mean ± standard deviation. Statistical significance was examined using two-way ANOVA (n = 3) and $p > 0.05$ between all groups.

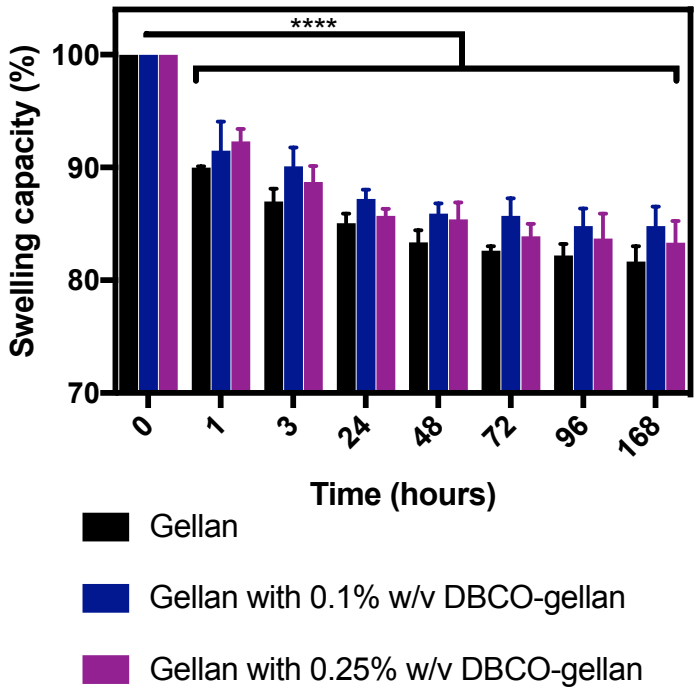

Figure S7. Swelling capacity over time of gellan hydrogels with increasing concentrations of DBCO-gellan in MSC growth media at 37 °C. Statistical significance was examined using two-way ANOVA (n = 4) and swelling capacities at all time points for a given hydrogel type were significantly different (**** $p < 0.0001$) from the initial non-swollen gellan hydrogel.

3. Cellular studies

Studies were conducted to investigate the interaction between MSCs and gellan. We first investigated the effect of the gellan polymer in solution on MSC viability using MSCs plated on tissue culture treated polystyrene. We than studied the effect of the covalent binding after seeding the cells on the surface of gellan hydrogels with increasing amounts of DBCO-gellan. Our approach was then compared with a conventional strategy based on RGD peptide polymer modification.

MSC culture

MSCs (passage 5 to 7) were expanded and used in MSC growth media consisting of low-glucose (1000 mg/L) DMEM supplemented with 10% v/v FBS, 1% v/v penicillin–streptomycin, and 4 mM L-glutamine at 37 °C in 5% CO_2. Media was completely refreshed every three days. For serum-free media experiments, the media was prepared analogous to the growth media omitting FBS.

MSC glycoengineering optimization

In order to determine the optimal conditions for engineering MSCs to express azide groups on the cell surface, MSCs were seeded on tissue-culture treated 96-well plates at a density of 12,000 cells/cm^2 in growth media. After 24 hours, the growth media was supplemented with an increasing concentration of Ac_4ManNAz (from 0 to 500 µM) and incubated for 24, 48, or 72 hours. After these incubation times a cell viability assay (CCK-8) was performed to assess the effect of Ac_4ManNAz on cell viability following the kit protocol. Briefly, CCK-8 solution was added to each well and the plates were incubated for 2 h at 37 °C. Absorbance of the wells was measured at 450 nm using a Cytation3 Plate Reader (BioTek, Winooski, VT). Positive controls of cells with no treatment and negative controls with no cells were included. Normalized cell viability was calculated using equation (S2)

$$\text{Normalized cell viability } (\%) = \frac{\text{Abs 450 nm (sample)} - \text{Abs 450 nm (negative control)}}{\text{Abs 450 nm (positive control)} - \text{Abs 450 nm (negative control)}} \times 100 \quad \text{(S2)}$$

The expression of azide groups over time and their reactivity towards DBCO was examined by culturing MSCs in growth media in the presence or absence of 50 µM Ac_4ManNAz after a defined incubation period (from 1 to 6 days). Cells were then treated with 50 µM carboxyrhodamine 110 DBCO and incubated in the dark for 1 hour. After this time, MSCs were washed three times with 0.01% v/v Tween 20 in 1× PBS, fixed with 4% paraformaldehyde in 1× PBS for 15 min at room temperature and washed again 9 times with 0.01% v/v Tween 20 in 1× PBS. The rhodamine fluorescence was quantified using a Cytation™ 3 plate reader (BioTek, Winooski, VT) daily over 6 days (excitation: 485 nm, emission: 523 nm). The effective reaction between DBCO and the azide on the cell surface was also confirmed using fluorescence microscopy (Nikon TiE inverted fluorescence microscope, Nikon Instruments, Inc., Melville, NY) using a 470/40 nm excitation and 525/50 nm emission (FITC) filter. For this experiment, MSCs were cultured in growth media in the presence of 50 µM of Ac_4ManNAz for 72 hours. Cells were then treated with or without 50 µM carboxyrhodamine 110 DBCO and incubated in the dark for 24 hours. After this time, MSCs were washed three times with 1× PBS and then imaged.

Investigating DBCO-gellan interactions with MSCs

To study the interaction between the DBCO-gellan and MSCs, cells were treated with the polymer at various concentrations. First, a cell viability assay (CCK-8) was performed and then MSCs were analyzed by fluorescence microscopy (Nikon TiE inverted fluorescence microscope, Nikon Instruments, Inc., Melville, NY) and flow cytometry (BD FACSAria Illu flow cytometer, Franklin Lakes, NJ). For cell viability studies, MSCs were first incubated with or without 50 µM Ac_4ManNAz for 72 hours and then seeded on tissue-culture treated 96-well plates at a density of 10,000 cells/cm^2 in growth media. After 24 hours, the growth media was supplemented with increasing concentrations of DBCO-gellan and incubated for an additional 24 hours. After this incubation time a CCK-8 assay was conducted as described in *MSC glycoengineering optimization*.

For fluorescence microscopy and flow cytometry studies, first DBCO-gellan was fluorescently labeled using carboxyrhodamine 110 azide. Briefly, DBCO-gellan and carboxyrhodamine 110 azide (1:0.1 DBCO to rhodamine molar ratio) were mixed in a 10% v/v DMSO aqueous solution and the reaction was left stirring at room temperature in the dark for 24 hours. Following this, the polymer was purified using a Pierce dye removal column followed by dialysis against water for 24 hours at room temperature using a 50 kDa molecular weight cut-off regenerated cellulose dialysis membrane. After lyophilization the conjugate was used without further purification.

For flow cytometry studies, MSCs with and without azide modification were seeded on tissue-culture treated 6-well plates (10,000 cells/cm^2) for 24 hours and then treated with DBCO-gellan or rhodamine labeled DBCO-gellan at a concentration of 25 μg/mL for 24 hours at 37 °C. A subset of DBCO-gellan treated MSCs were subsequently incubated with 50 μM carboxyrhodamine 110 azide for 2 hours. Controls of azide and non-azide modified MSCs with no further treatment and those treated for 2 hours with 1 or 50 μM carboxyrhodamine 110 azide were included. After incubation, MSCs were detached via trypsin-EDTA, washed with 1× PBS and analyzed by flow cytometry using a 488 nm (FITC) laser on a 4-laser, 19-parameter BD FACSAria Illu flow cytometer (Franklin Lakes, NJ).

Investigating MSC interactions with gellan hydrogels

MSC interactions with gellan hydrogels fabricated with DBCO-gellan or RGD-gellan were monitored using both confocal microscopy imaging (A1R confocal laser microscope, Nikon Instruments, Inc., Melville, NY) and cell viability analysis. For all hydrogels monitored, azide and non-azide modified MSCs were seeded on gellan hydrogel surfaces (10,000 cells/cm^2) for 24 hours. For confocal microscopy, MSCs on hydrogels in cover-glass bottom chamber slides were fixed and stained for F-actin and nuclei. Briefly MSCs were fixed with 4% paraformaldehyde in 1× PBS for 15 min at room temperature (RT), permeabilized with 0.1% v/v Triton X-100 in 1× PBS for 5 min RT, and blocked with 10% v/v normal goat serum (NGS) in 1× PBS for 30 min RT. Cells were then stained for F-actin (Alexa-Fluor 594-phalloidin 1:100 in 1% v/v NGS in 1× PBS for 90 minutes RT) and for nuclei (2 μM DAPI in 1× PBS for 7 min RT). For vinculin staining, MSCs were first permeabilized for 1 minute with cytoskeletal buffer (0.5% Triton X-100, 300 mM sucrose, 50 mM sodium chloride, 10 mM HEPES, and 6.4 mM magnesium chloride hydrate), then fixed and treated with 1% v/v NGS, as described above. Cells were then stained for vinculin (monoclonal anti-vinculin-FITC antibody 1:100 in 1% v/v NGS in 1× PBS overnight at 4 °C), for F-actin (Alexa-Fluor 594-phalloidin 1:100 in 1% v/v NGS in 1× PBS for 90 minutes RT), and for nuclei (2 μM DAPI in 1× PBS for 7 min RT). Following all staining, cells were imaged via confocal microscopy. For cell viability, MSCs were treated with CCK-8, as described in *MSC glycoengineering optimization* and viability was examined by measuring absorbance at 450 nm using a Cytation3 Plate Reader (BioTek, Winooski, VT). Cell viability was normalized to the signal obtained from cells seeded on hydrogels lacking DBCO treated with CCK-8. Equation (S3) was used to calculate the normalized cell viability:

$$\text{Normalized cell viability} = \frac{\text{Abs 450 nm (sample)} - \text{Abs 450 nm (negative control)}}{\text{Abs 450 nm (unmodified gellan hydrogel with cells)} - \text{Abs 450 nm (unmodified gellan hydrogel without cells)}} \quad \text{(S3)}$$

Here, the negative controls were the hydrogels without any cells seeded.

Investigating protein interactions with gellan hydrogels

To study the interaction between gellan and extracellular matrix proteins, gellan hydrogels with or without 0.1% DBCO-gellan were incubated with fibronectin HiLyte 488. Hydrogels (50 µL) were prepared in wells of 96-well plates and 10 µL of this fibronectin at 0.1 mg/mL was deposited on the surface of each hydrogel. The solution was evaporated at room temperature for 60 minutes in the dark and then rinsed three times with 1× PBS. The hydrogel surface was then imaged using fluorescence microscopy (Nikon TiE inverted fluorescence microscope, Nikon Instruments, Inc., Melville, NY) using a 470/40 nm excitation and 525/50 nm emission (FITC) filter.

Statistical analysis

All experiments were conducted in triplicate at minimum. Results are presented as mean ± standard deviation where appropriate. Statistical significance was evaluated using Prism 7 (GraphPad) with unpaired t-test and two-way analysis of variance (ANOVA). Tukey's post hoc analysis was performed for ANOVA tests ($\alpha = 0.05$). Note, $p < 0.05$ was considered statistically significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Image J (National Institutes of Health) was utilized to examine all microscopy images.

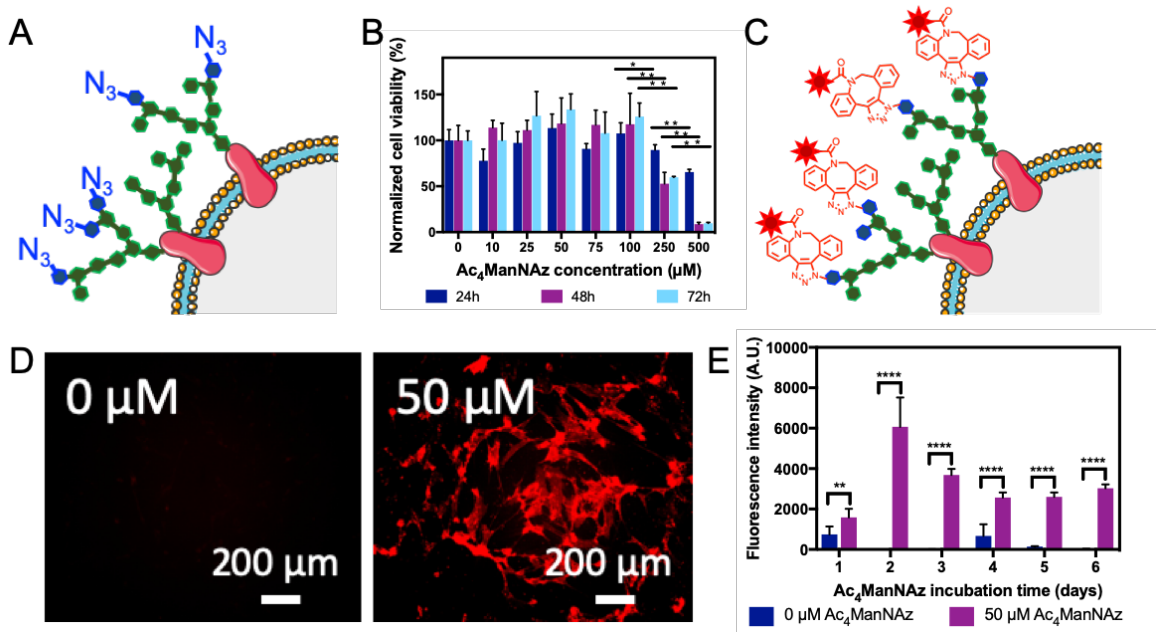

Figure S8. A) Schematic of cell surface functionalized with Ac_4ManNAz. B) Effect of Ac_4ManNAz concentration on MSC viability over time. C) Schematic of cell surface with DBCO-rhodamine clicked on the expressed azides. D) Fluorescence microscope images of MSCs incubated first with Ac_4ManNAz (50 µM) for 72 hours and then with (right) or without (left) 50 µM DBCO carboxyrhodamine 110 for an additional 24 hours. E) Azide expression on MSCs over time quantified by measuring rhodamine fluorescence after incubating azide modified MSCs with carboxyrhodamine 110 DBCO. Statistical significance was examined using unpaired t-test (n = 3, ** $p < 0.01$, **** $p < 0.0001$).

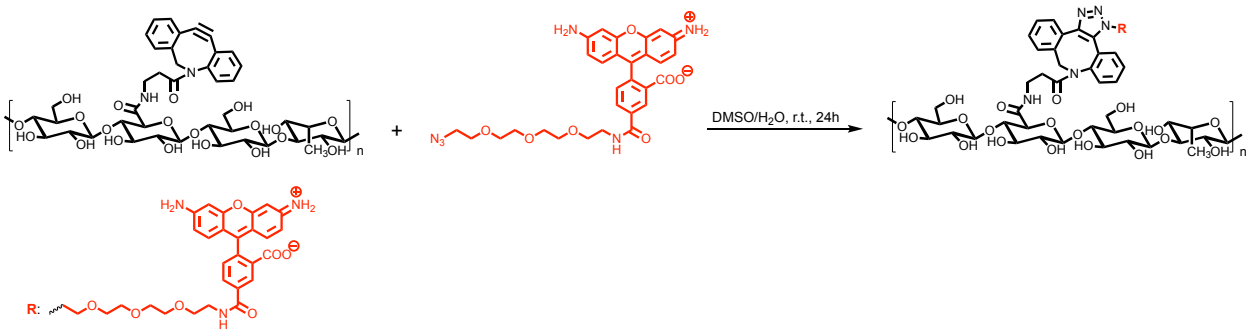

Figure S9. Reaction scheme of DBCO-gellan functionalization with rhodamine.

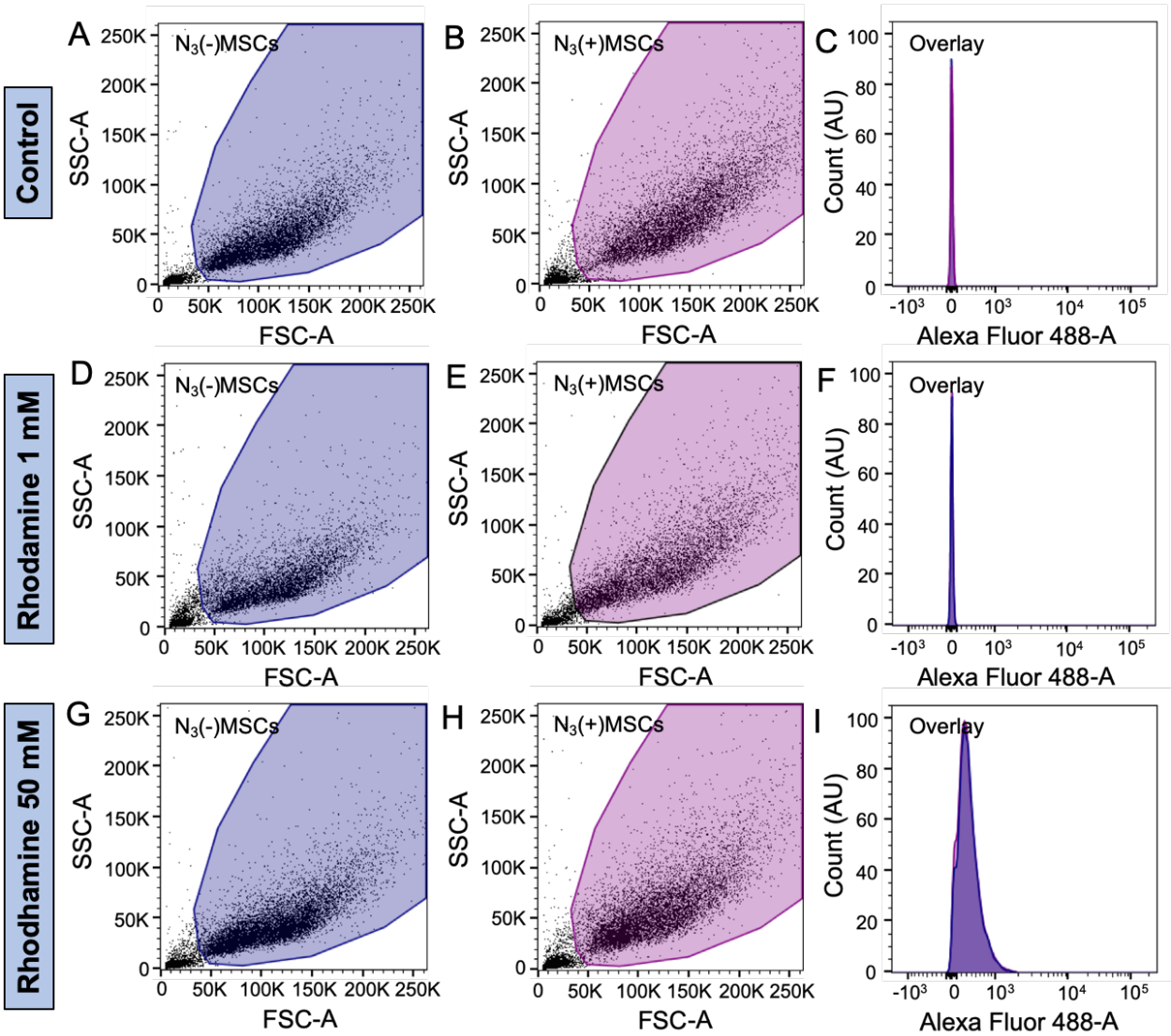

Figure S10. Flow cytometry dot plots of MSCs not treated with DBCO-gellan for non-azide modified MSCs (N_3(-)MSCs) (A, D, and G) and azide modified MSCs (N_3(+)MSCs) (B, E, and H) and overlay of fluorescence intensity histograms of the gated regions (C, F, and I). First row: untreated cell controls (A, B, and C). Second row: treatment with 1 mM carboxyrhodamine 110 azide (D, E, and F). Third row: treatment with 50 mM carboxyrhodamine 110 azide (G, H, and I).

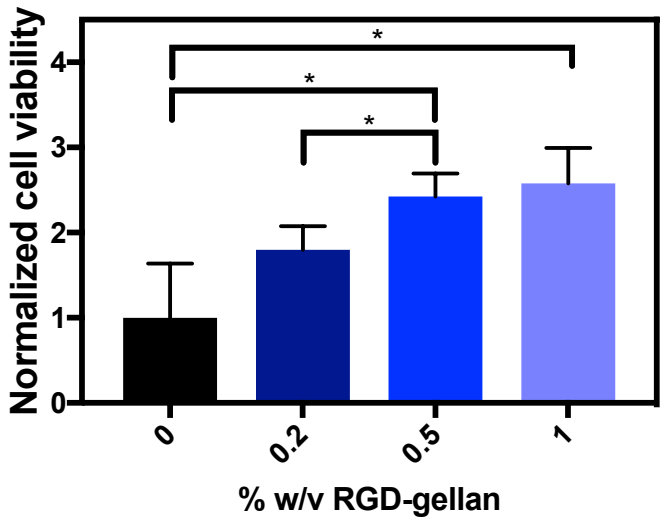

Figure S11. Normalized cell viability of MSCs seeded on 2% w/v gellan hydrogel surface with increasing concentration of RGD-gellan. Statistical significance was examined using unpaired t-test (n = 3, * $p < 0.05$).

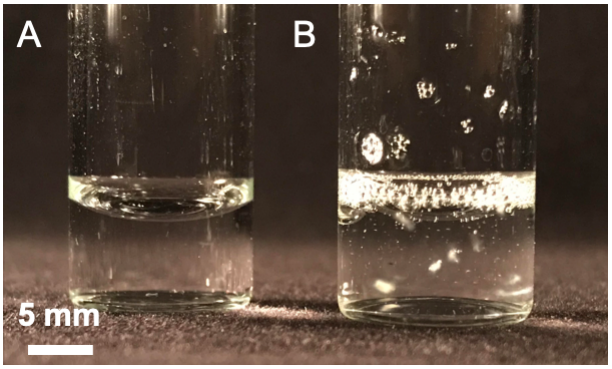

Figure S12. Assessing DBCO-gellan aggregation in solution. Unfunctionalized gellan (A) versus DBCO-gellan (B) in solution at a concentration of 2 mg/mL. The DBCO-gellan solution contains heterogeneous aggregates throughout.

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